STUDIES ON THE PHYSIOLOGY OF THE EUGLENOID FLAGELLATES. I. THE RELATION OF THE DENSITY OF POPULATION TO THE GROWTH RATE OF *EUGLENA*.

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Introduction.

The relation of volume of culture medium to the rate of growth of ciliates has been investigated by a number of workers during the last two decades, but up to the time of this investigation no similar experiments on the green flagellates had been attempted. The object of the present investigation, therefore, was to determine whether or not such a relationship exists in growth of *Euglena,* and also whether or not there is evidence for an "allelocatalytic effect," such as described by Robertson (1921*b,* 1922) for ciliates.

For this series of experiments *Euglena* sp. was chosen for its ability to grow rapidly in "autotrophic" media. Also, in such a medium the food supply of an organism is much less affected by bacterial activities than if a large amount of organic material were present. The first experiments were carried out in the same manner as were previous isolation experiments with ciliates (Woodruff, 1911, 1913; Robertson, 1921*a, b,* 1922; Cutler and Crump, 1923*a, b,* 1924, 1925; Greenleaf, 1926; Myers, 1927; Yocom, 1928). This method was discarded later in favor of a mass method in which variations in the concentration of organisms were substituted for variations in volume of the medium.

This investigation was carried out at the suggestion of Doctor R. P. Hall, and the writer wishes to express his gratitude for suggestions and aid in the preparation of the present paper. The writer also wishes to thank Professor Asa C. Chandler for the use of the Biological Laboratory of the Rice Institute during the summer of 1928.

Historical Survey.

The earliest observations on the relation of volume of culture medium and division rate were those of Woodruff (1911) on two species of *Paramecium* (*P. caudatum* and *P. aurelia*), which showed a more rapid division rate in larger than in smaller volumes of the same culture medium. Woodruff suggested that this was due to a more rapid accumulation of waste products in cultures of smaller volume, and in later observations (Woodruff, 1913) he showed that waste products of any one species (of *Paramecium, Stylonychia, Pleurotricha*) actually depressed the division rate of the same species, although apparently without effect on other species.

The next investigations in this field were carried out by Robertson (1921*a, b,* 1922, 1924*b, c*) who found that when two ciliates were isolated into the same drop of culture medium the division rate was higher (sometimes sixteen times as great) than if a single cell were isolated into a drop of the same size. This difference in division rate is, according to Robertson, due to the mutual contiguity of the cells, and has been designated by him as an "allelocatalytic effect," caused by a growth-catalysing substance liberated from the nucleus into the cytoplasm and thence into the surrounding medium during nuclear division. This theoretical catalyst is called by Robertson an "autocatalyst of growth." The amount of this catalyst present in isolation cultures would depend upon the number of cell divisions that had taken place; therefore, the culture with the greater number of dividing cells would have a greater concentration of catalyst, and the division rate in these cultures should be correspondingly greater.

Cutler and Crump (1923*a, b*) found no evidence for the occurrence of allelocatalysis in cultures of *Colpidium* with 1, 2, 3, and 4 initial organisms in volumes that varied from 0.5 to 8.5 cubic millimeters, or in mass cultures with concentrations of 100–8,000 organisms per cc. They pointed out that their results as well as those of Robertson, might be explained by the presence of a larger food supply in the cultures showing a higher division rate.

Greenleaf (1924, 1926), in a large number of experiments in which the volume of medium was varied from two to forty drops,

found that two species of *Paramecium* (*P. aurelia, P. caudatum*) and *Pleurotricha lanceolata* showed a higher division rate in larger volumes of medium, and also that in cultures of *Stylonychia pustulata* the division rate of a single animal was greater than that of either of a pair of animals when introduced into the same volume of medium.

Calkins (1926) made a sixty-day test of *Uroleptus mobilis* with one, two, three, and four individuals per drop. The cultures with one initial organism showed a higher division rate than those with two organisms, and those with two organisms a higher division rate than those with three and four.

Myers (1927), in a long series of experiments, found that the division rate of *Paramecium* decreased with the density of population, and that a certain minimum volume (0.2–0.4 cc., or 4–8 drops) of "flourishing" hay infusion was necessary for a single *Paramecium* to multiply at its maximum rate.

Yocom (1928) found that in *Oxytricha* four-drop cultures had a 14 per cent. higher division rate than ten-drop cultures. In each series of experiments, however, there were some cases in which the division rates of the ten-drop cultures were equal to or higher than those of the four-drop cultures. Only by averaging a number of cases could he show an appreciable difference. He accredits this difference to the earlier attainment of a high concentration of autocatalyst in the cultures of smaller volume.

Petersen (1929) has found that within certain limits a higher division rate of *Paramecium* is present in cultures of smaller volume and in cultures containing a larger number of ciliates. Some of her results, however, tend to contradict Robertson's theory.

In reviewing the work on volume-division rate relations in Protozoa one finds that Robertson's theory is supported only by the work of Yocom and by some of the results of Petersen. On the other hand, the experiments of Woodruff, Cutler and Crump, Greenleaf, Calkins, Myers, and some of the results of Petersen, furnish evidence which contradicts the theory of an autocatalyst.

Material and Methods.

Early in the summer of 1928 water was collected from several ponds in the vicinity of Houston, Texas, including that known

as the "Biology Pond" of the Rice Institute. This was placed in aquarium-jars and gave rise to mixed euglenoid cultures. An autotrophic medium similar to those used by Mainx (1928) and Günther (1928) was prepared as follows:

NH_4NO_3	10 gms.
KH_2PO_4	5 gms.
$MgSO_4$	1 gm.
H_2O (distilled)	1000 cc.

This stock solution was diluted ten times, brought to pH 7.0 with Na_2Co_3, and then boiled before being used. Culture material was centrifuged, the supernatant liquid poured off, and the synthetic medium added. By repeated centrifugings and washings over several days the organic content was reduced to a minimum. Some of the *Euglena* present continued to grow and gave rise to a mass culture in the autotophic medium. By means of a mouth-controlled suction pipette single specimens of *Euglena* sp. were isolated into fresh medium in depression slides. All *Euglena* used in subsequent experiments were descendants of a single specimen isolated at this time. The depression slides were kept on racks in dessicators and large petri dishes half filled with water.

In order to determine the relation, if any, of volume of culture medium to division rate, sister cells were isolated into different amounts of medium. On account of the small size of the flagellates, the most practicable volumes for such cultures ranged from two to six drops. In all, twenty-two pairs were started at various times. In every case a flagellate was chosen at random from the mass culture derived from a single *Euglena*. Each organism was isolated into a depression slide. After the first division the daughter cells were washed twice in three drops of medium, then placed in separate depressions on another slide and immediately covered with fresh medium—one with two drops, the other with six. For each pair of cultures, counts were made daily and the numbers present recorded.

At the end of ten days, three pairs of cultures showed from two to three and a half times as many organisms in the cultures of smaller volume. In one pair, however, three times as many were present in the culture of larger volume. In another instance the numbers were about equal. The rest of the cultures were dis-

carded on account of excessive evaporation. Such results are by no means conclusive, since they indicate a marked variability in the division rate of this flagellate.

The isolation method in this case, therefore, seemed to be unreliable, and was abandoned for the following reasons: (1) The mortality rate was high, due to the evaporation of the medium. (2) Evaporation increased the salt concentration, and consequently the osmotic pressure of the medium. (3) The practicable range of variation in volume was limited (two to six drops). (4) The results obtained were contradictory. In many cases, synchronous division did not occur in pure lines derived from sister cells left undisturbed in the same depression slide. (5) An extremely large number of experiments would be necessary in order to obtain growth curves that would approach accuracy. (6) Bacterial counts could not be made for such cultures without greatly altering the volume of the medium. In order to avoid as many as possible of these difficulties the mass culture method was adopted.

The same pure line used in isolation cultures was grown in mass cultures in the autotrophic medium already described. By repeated centrifuging and washing, cultures with low bacterial counts were obtained. These were concentrated in centrifuge tubes, and various dilutions were made. In 18×40 mm. test tubes, 20 cc. of each dilution was kept at room temperature under a constant source of light, consisting of a battery of three 100-watt globes (frosted on the inside). In series I., II., III., and IV., the cultures were protected from the heat by a wind tunnel with top and bottom of thin plate glass. An eight-inch fan removed practically all of the surplus heat. The battery of lights was above the tunnel and the cultures below. Mirrors were placed both above the lights and below the cultures in order to insure as uniform a distribution of light as possible. The number of organisms per cc. was counted at the beginning of the experiment and at convenient intervals thereafter. Bacterial counts were made by plating according to the standard method used in water analysis.

Bacterial counts were made on each dilution at the beginning and at the end of each series and in some cases at the end of thirty-six hours also. Initial counts were always low, ranging from 200 to 1,500, and in a few cases to 3,000 per cc. Thirty-six hour

counts were much higher, ranging from 20,000 to 50,000 per cc. Final counts ranged from 30,000 to several hundred thousand per cc. Since the effect of bacterial products on the rate of division of *Euglena* is unknown in this case the significance of the counts can not be stated. It is worth noting, however, that the larger number of bacteria after the first day did not result in an increased division rate. On the contrary, the division rate in cultures of *Euglena* sp. decreased continuously after the first few divisions, thus indicating that their growth was either hindered or else unaffected by the increase in number of bacteria. If bacteria hindered the division rate, this might possibly be effected in one or both of two ways—by utilizing and thereby decreasing the food supply of the euglenoids, or by excreting products of an injurious nature into the medium. However, there is no evidence supporting either possibility, or indicating that the bacteria affected the division rate of the flagellates in any way.

In series V. and VI., Carrel tissue culture flasks were used instead of test tubes. The Carrel flasks assured a much more uniform distribution of light than did the test tubes. The flasks were only half filled in order to allow air circulation. They were suspended in a wire rack and partially immersed in a Freas Water Thermostat (of the small type, accurate to .1° C. for long periods of time) at 27° C.

A Sedgwick-Rafter counting chamber of 0.5 cc. capacity and a Whipple micrometer were used for counting. The method of counting was, in general, that described by Whipple (1927) for counting plankton. Each tube or flask was sampled twice, and usually seventy-two 1 mm. squares were counted and averaged for each sample. If the counts for the two samples differed as much as ten per cent., a third count was made. These three counts were then averaged, and the result was taken as the count for the tube or flask. When duplicate flasks were used each flask was sampled twice, and a count was made for each. The counts for the two flasks were then averaged to find the final count for the dilution in question. In determining the initial concentrations, the average count of five samples was taken in order to insure as great accuracy as practicable. Pipettes for filling the counting chamber were washed thoroughly, autoclaved or boiled, and then dried before being used.

In all cases the concentration of flagellates has been expressed as thousands per cc. or decimal fractions thereof. For example, a concentration of 540 individuals per cc. is expressed as .54 thousand per cc., or merely as .54. No large series of counts was undertaken to determine the exact percentage error, but it is believed that the error is rather low. For example, one set of initial counts was as follows: .87, .90, .87, .91, .92, with an average of .894. Assuming that the flasks were well shaken before sampling, the standard error of such a system of counting is plus or minus the square root of the total number counted (Fisher, 1925, p. 59). In the set of initial counts mentioned above this would be $\sqrt{321}$ or $\pm$ 18, giving as the total corrected number 321 $\pm$ 18, or a concentration of .894 $\pm$ .047. The probable error is always .67499 times the standard error, and in this case is .67499 $\times \pm$ .047, or $\pm$ .0317, making the concentration .894 $\pm$ .032. This is a probable error of 3.52%. However, since the greatest deviation of the individual counts from the mean is .026 in the series mentioned, it is fairly certain that the error is very much less than the standard and is well within the probable error. Concentrations of less than .5 were not counted because of the large number of squares that would have to be counted in order to insure a fair degree of accuracy.

It may seem that four hundred organisms per cubic centimeter is a high concentration, and that from the beginning the cultures might suffer from crowding. However, if the total volume of the organisms is compared to the volume of the medium, it is seen that this is not the case. Taking the radius of a rounded organism as approximately eleven microns, we may calculate its volume to be 4.189×11^3, or approximately 5,575 cubic microns. Since one cubic centimeter is equal to 10^{12} cubic microns, the ratio of the volume of 450 *Euglena* to that of their containing fluid is $\frac{450 \times 5.575}{10^{12}}, \frac{2{,}608{,}750}{10^{12}}$, or approximately 1 : 383,300. This means that in the most concentrated cultures of all except the first series of experiments, the initial volume of the flagellates was approximately 1/383,300 that of their surrounding medium, while in the most dilute cultures the volume ratio of organism to medium was 1 : 3,833,000.

Experimental Results.

Series I.

On October 14 the organisms were washed and centrifuged six times. On October 15 they were centrifuged five times before the experiment was started. Since the centrifuge tubes were filled to approximately 18 cc. and about 17.75 cc. of supernatant liquid was poured off each time, the concentration of any catalyst of growth, if present, was reduced to approximately 1/72 $\left(\frac{18 - 17.75}{18}\right)$ of its previous concentration with each washing. Two consecutive washings would reduce the autocatalyst to approximately 1/5,000 of its original concentration; five washings to 1/1,900,000,000. The organisms, after washing, were concentrated by centrifuging and then counted. The concentration was found to be 39.2 thousand per cc. Various numbers of organisms were placed in five culture tubes, which were then filled with medium to a volume of 20 cc. The concentration was then determined for each tube. The initial count in tube number 1 was 0.42; in tube 2, 0.82; tube 3, 1.9; tube 4, 5.0; tube 5, 9.5.

Counts were made subsequently at intervals of 24–36 hours. The average counts were accurately plotted against time, as shown in figure 1. In Fig. 2 the same curves are repeated, but in this case x/x_0 (where x represents the initial number, *e.g.*, .42 in tube number 1) is plotted against time; in other words the curves shown in Fig. 1 are all reduced to unity in Fig. 2. It is readily seen that, in general, the increase in number per initial organism was much greater in the more diluted cultures (Fig. 2). An objection to the concentration used is readily seen in the fact that the more concentrated cultures were initially quite crowded; hence they were probably restricted sooner and more severely by limiting factors than were the more diluted ones. Therefore, other series of experiments were started in which all dilutions were higher than that of culture 1 of series I. It was hoped that these would yield a set of curves in which the limiting factors would be negligible for the first few days. The results of such experiments are described in series II., III., IV., V., and VI.

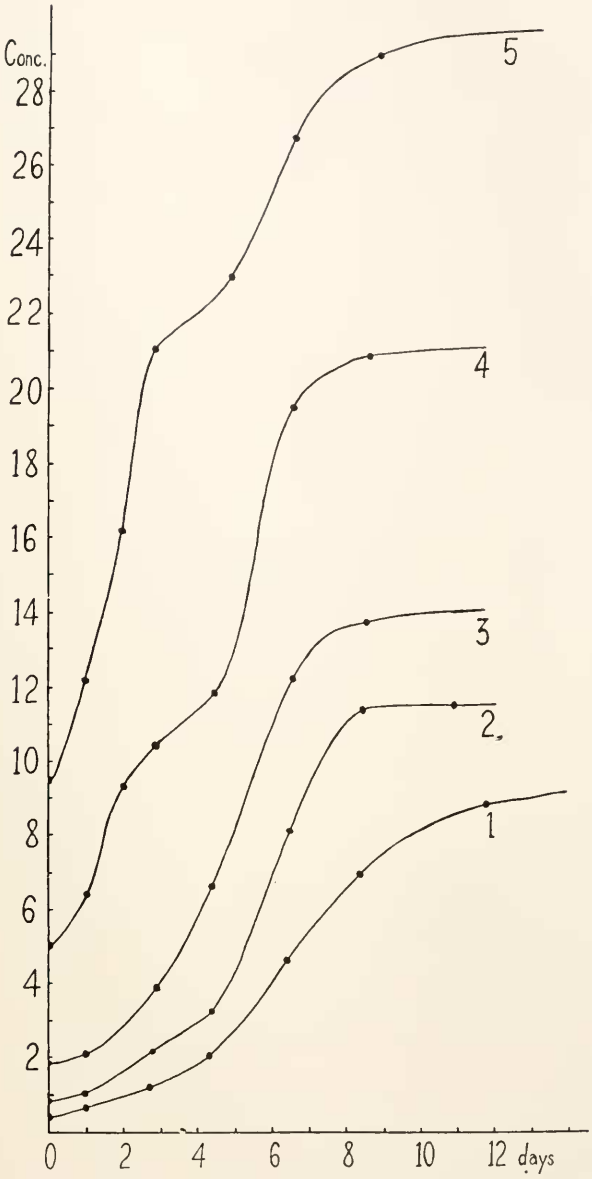

FIG. 1. Graph showing concentration of organisms (in thousands per cc.) plotted against time for series I. Curves 1, 2, 3, etc., represent the numbers present in tubes 1, 2, 3, etc., respectively. Dots represent observed points.

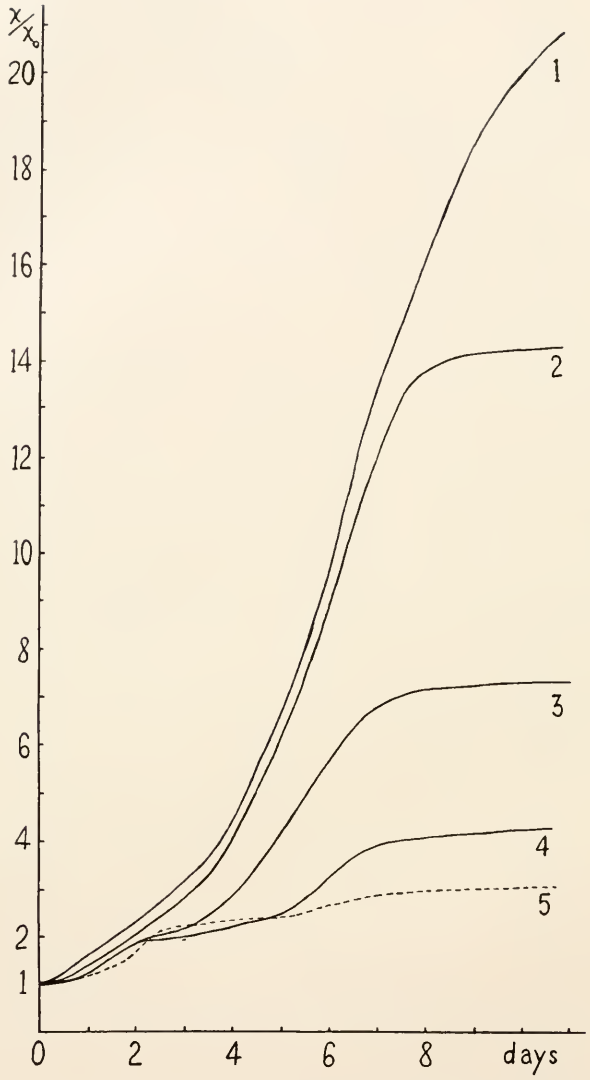

FIG. 2. Graph showing x/x_0 (x_0 being the number at any time t; x_0, the initial number) of the curves of Fig. 1 plotted against time. Note that the order of the curves is reversed. The most concentrated culture (curve 5) shows the smallest increase (with a ratio of 3:1), while the most dilute (curve 1) shows the largest increase (ratio of 21:1). The others show intermediate ratios varying in reverse order with their original concentrations. These curves thus indicate higher rates of division in the less concentrated cultures.

Series II.

The organisms were centrifuged a number of times over several days, and three times just before the experiment was started. Three dilutions were used, and they were maintained in duplicate, the tubes being designated as 1*A*, 1*B*, etc., respectively. One concentration was made and counted. It was found to be .555.

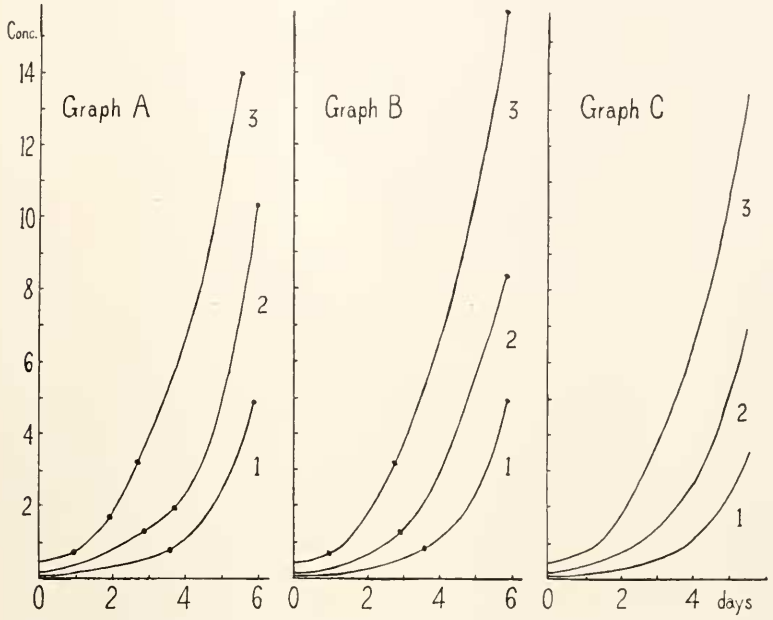

FIG. 3. Graphs showing concentrations for series II. plotted against time.
Graph *A*.—Curves 1, 2, and 3 show numbers in cultures 1*A*, 2*A*, and 3*A*, respectively. Dots indicate observed points.
Graph *B*—Curves 1, 2, and 3 show numbers in cultures 1*B*, 2*B*, and 3*B*, respectively. Dots indicate observed points.
Graph *C*—A combination of graphs *A* and *B*, obtained by averaging corresponding values for the curve of the two preceding graphs.

The fact that graphs *A* and *B* are very similar and that the values of x for any dilution at any given time do not vary much in the two sets of cultures is a demonstration of the efficiency of the method used. Initial numbers were the same for the corresponding curves, conditions were the same for the two sets of cultures, and the resulting curves are very similar.

Twenty cc. of this was placed in tubes 3*A* and 3*B*. Part of the remainder was diluted one to three with sterile medium to make a concentration of .14. Twenty cc. of this was placed in tubes 2*A*

and 2*B*. The remainder was diluted one to nine to make a concentration of .055, of which 20 cc. was placed in tubes 1*A* and 1*B*. The results are shown in Fig. 3. Laboratory conditions prohibited the continuance of the experiment more than six days. Fig. 4

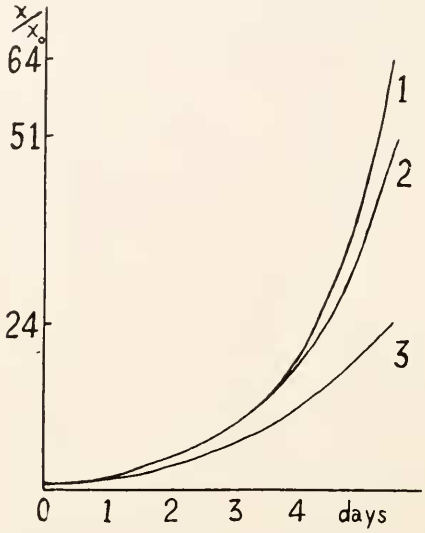

FIG. 4. Graph showing x/x_0 (x being the number at any time t; x_0, the initial number) of graph *C*, Fig. 3, plotted against time. Note the higher relative rate of increase (ratio 64:1) in the more dilute culture represented by curve 1, as compared with the most concentrated culture represented by curve 3 (ratio 24:1). This is directly opposed to any theory of an autocatalyst.

shows x/x_0 of the preceding curve (Fig. 3) plotted against time. The higher division rates of the more dilute cultures are obvious, especially after the first few days when the accumulative differences become quite large.

Series III.

The organisms were centrifuged at least a dozen times during several days and three times just before the beginning of the series. The flagellates were counted. The concentration in tubes 3*A* and 3*B* was .35. The dilution was 1 to 3 for tubes 2*A* and 2*B* and 1 to 9 for tubes 1*A* and 1*B*, as in the previous series. At the end of the first 24 hours (Fig. 5) the count for the most concentrated cultures was .73, and at the end of 48 hours was 1.32, and at the

end of 72 hours was 2.07 as shown in Fig. 5. At the end of 72 hours the count for the second dilution (tubes $2A$ and $2B$) was .51. Due to an accident no count for the highest dilution could be obtained. For this reason the series was discontinued. The initial concentration in tubes $2A$ and $2B$ was $\frac{1}{4}$ of that in tubes $3A$ and $3B$. This ratio was still maintained at the end of the third day when counts were .51 and 2.07.

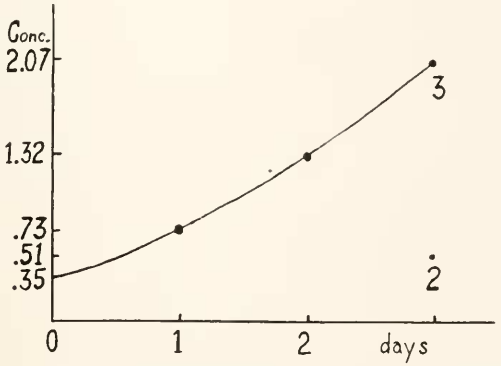

FIG. 5. Graphs showing concentrations in series III. plotted against time. Due to an accident data for one curve and for only one point on another curve was obtained. These were dilutions 3 and 2. The ratio of the initial concentrations was 4 : 1, and the ratio of their final concentrations at the end of the third day was 2.07 : .51—practically the same as the original. This experiment shows no difference in division rate in the two sets of cultures and is opposed to the theory of an autocatalyst.

Series IV.

The flagellates were centrifuged several times, dilutions were made exactly as in series II. and III., and cultures were maintained in duplicate. The initial count for the most concentrated cultures (dilution 3) was .894. The concentration of dilution 2 was .233; that of dilution 1 was .089. The final counts at the end of seven days on the three sets of cultures (six tubes) showed no large difference of growth rate. The final counts were 0.61, 1.50, and 6.68 for dilutions 1, 2, and 3 respectively; whereas their initial ratio was 1 : 2, 5 : 10.

From this it can be seen that the cultures have maintained approximately their original ratio of concentrations. A slight gain is seen in the most concentrated (dilution 3) over the more dilute

cultures (dilutions 1 and 2). Dilutions 1 and 2, however, show the same ratio at the end as at the beginning of the experiment. The conclusion to be drawn from this series is that the division rate in the more concentrated cultures is at least equal to or perhaps slightly greater than that in the more dilute cultures.

Series V.

The organisms were washed at least a dozen times over a period of several days and three times just before the experiment was started. Dilutions were made exactly as in the previous series, the

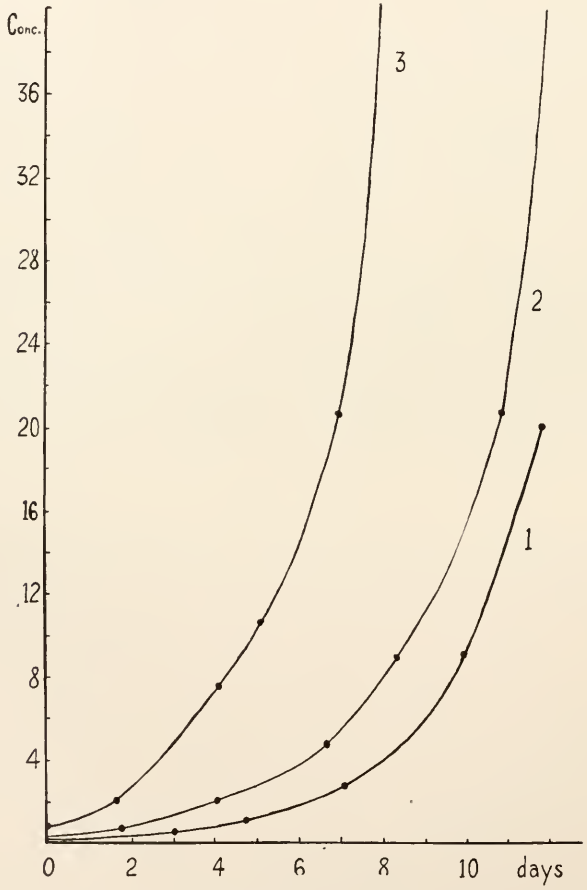

FIG. 6. Graph showing the concentrations of series V. plotted against time.

initial concentrations being .066, .165, and .66 for dilutions 1, 2, and 3 respectively. Duplicate cultures were maintained as in the previous series. The results obtained are shown in Fig. 6, and the curves are reduced to unity in Fig. 7. The increase in numbers was proportionately higher in the more dilute cultures.

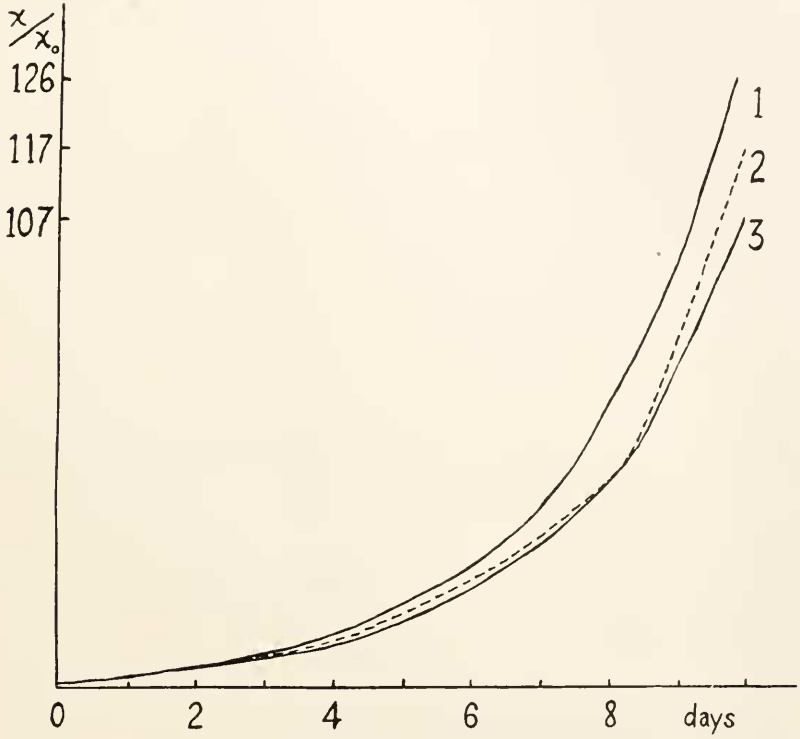

FIG. 7. Graph showing x/x_0 (x being the number present at any time, t; x_0 the initial number) of Fig. 6 (series V.) plotted against time. Note that the order of the curves is reversed from that shown in Fig. 6, denoting a greater relative increase in the most dilute culture (curve 1) at the end of the tenth day (ratio of 126: 1) than in the most concentrated culture (curve 3, with a ratio of 107: 1). This is directly opposed to the theory of an autocatalyst.

Series VI.

The organisms were washed, and dilutions were made as in previous series. Initial concentrations were .055, .14, and .55 in dilutions 1, 2, and 3 respectively. These were maintained in duplicate. The results are shown in Fig. 8, and the curves are reduced

to unity in Fig. 9. The rate of increase in number was proportionately the same in the lowest and in the highest dilutions for the first seven days. Then the most dilute showed a larger increase than the most concentrated. The intermediate dilution

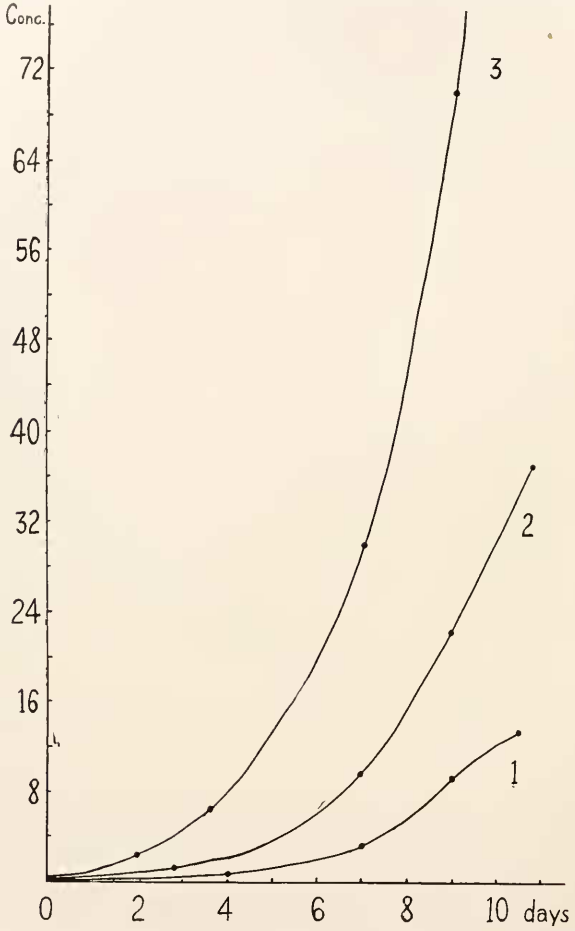

FIG. 8. Graph showing the concentrations in series VI. plotted against time.

(dilution 2) showed a higher increase on the sixth, seventh, and eighth days, a lower increase on the ninth and tenth days, and a higher increase on the eleventh day than the other dilutions. The reason for this is not known. Since no large differences were

observed in the relative amount of increase and since the minor differences were contradictory, the only conclusion to be drawn is that the concentration of organisms had no significant effect on the division rate.

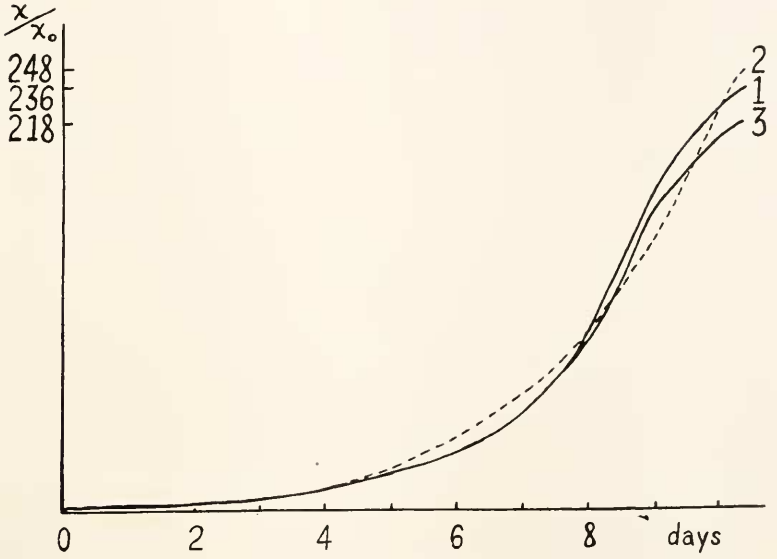

FIG. 9. Graph showing x/x_0 (x being the number present at any time t; x_0, the initial number) of the curves in Fig. 8 (series VI.) plotted against time. A difference in relative amount of increase becomes apparent between the lowest and highest dilutions after seven and one half days, the final ratios being 236:1 for the highest dilution (curve 1) and 218:1 for the lowest dilution (curve 3). The relative increase of the intermediate dilution is somewhat erratic, being higher during the sixth, seventh, and eighth days, lower during the ninth and tenth, and higher on the eleventh. These results are, of course, contradictory and indicate only that there is no large difference in division rate between cultures of low and high dilution.

DISCUSSION.

As a whole, the results of the writer's experiments with *Euglena* offer no evidence of any allelocatalytic effect. In most of the cultures, especially after the first few days, a significant difference in the opposite direction was apparent. This may be easily explained on the basis of food supply, for the more concentrated cultures would certainly use more food than the more dilute ones, leaving a lower concentration of foodstuffs in the surrounding

medium and thus directly depressing the division rate. The more concentrated cultures would also contain a higher concentration of waste products. The most probable explanation of a higher division rate in the more dilute and a lower rate in the more concentrated cultures is that the flagellates in the higher concentrations were hindered by a scant supply of food or a relatively large amount of waste products, or more likely by both these factors.

This conclusion is in accord with the experiments of Woodruff (1911, 1913) who found that waste products of one species of ciliate inhibited the division rate of the same species; and also with the observations of Greenleaf (1924, 1926) that in several ciliates a higher division rate occurred in larger volumes of medium, and that no allelocatalytic effect was present. These results are also in agreement with those of Cutler and Crump (1923*a*, *b*, 1924, 1925) for both isolation and mass cultures of *Colpidium*. Cutler and Crump (1924, 1925) explained all their results as being due to differences in food supply between large and small volumes, and their data is directly opposed to any theory of an autocatalyst.

Petersen (1929) points out that some of Cutler and Crump's (1923*a*) results indicate a statistical difference in division rate which is in favor of an allelocatalytic effect—a fact which is admitted by the authors. However, this is true only in a limited range of volume (0.5–2.5 cubic millimeters) and only when their one-animal cultures are compared to their two-, three-, and four-animal cultures. If the theory of allelocatalysis is applicable, one should expect to find differences between two- and three-, and between three- and four-animal cultures, and a very marked drop in division rate when single individuals were isolated into very large volumes. Cutler and Crump's (1923*a*, *b*) results are exactly opposite to this. If their data for two-, three-, and four-animal cultures in 2.5 to 8.5 cubic millimeters are considered, a higher division rate is obvious in the larger volumes within this range. If all volumes from .5 to 8.5 cubic millimeters are considered, however, the average division rates are about the same, regardless of whether the initial number of ciliates was one, two, three, or four. This indicates a total absence of any allelocatalytic effect. It is difficult to understand why Petersen should draw her conclu-

sions only from Cutler and Crump's small-volume cultures, when data from their other volume variations are directly contradictory.

Furthermore, in a paper which Petersen has apparently overlooked, Cutler and Crump (1924) have demonstrated a linear relationship between the average division rate and the number of initial organisms when the initial numbers are plotted in geometrical progression. In other words, they found a positive correlation between volume of medium and division rate, whereas a negative correlation between volume and division rate would be necessary to support Robertson. This relationship is shown in both isolation and mass cultures. In Cutler and Crump's earlier paper (1923*b*) this correlation was masked by the fact that the initial numbers were not plotted in geometrical progression but were given in tabular form. Even when plotted in arithmetical series they show a correlation coefficient of — .37 between division rate and initial concentration of organisms in mass cultures. This, of course, would correspond to a positive correlation of division rate and volume of medium per organism. Although this is a low correlation coefficient, it is significant because it exceeds its probable error by eight and a half times, and any correlation coefficient which exceeds its probable error may be deemed significant. In view of this overwhelming evidence it is certainly impossible to accept Petersen's (1929) interpretation of Cutler and Crump's (1923*a*, *b*, 1925) results. Their own conclusion (1924, 1925), that the division rate is higher in cultures with a lower concentration of organisms, is well founded and supported by their experiments, and is directly opposed to any theory of an autocatalyst.

Myers (1927 found that the division rate of *Paramecium* decreased with the density of population. Peterson (1929), however, shows that by selecting certain data from Myers' paper she could find some evidence for allelocatalysis. Myers, by averaging five lines, computed the mean generation time to the first division for 1, 2, 4, and 8 individuals isolated into 2, 4, 8, and 16 drops of medium. He performed three series of experiments—one series each with fresh, one-day-old, and two-day-old medium. In most cases the time to the first fission increased with the density of population. The two sets of experiments selected by Petersen are the sixteen drop cultures from the one- and two-day infusions,

although the ten other similar sets of experiments show more definite and exactly opposite results. These two sets, however, do show a difference of 2.4 hours in time to the first fission between one- and eight-animal cultures. Myers recorded the number present at the end of six, twelve and twenty-four hours in each of the five lines, thus determining the generation time of each line as six, twelve, or twenty-four hours. The mean generation time was then obtained by averaging the five lines. It is very unlikely, however, that these mean generation times are accurate because the intervals between counts (six hours) were approximately equal to the average generation time. Therefore it was impossible to obtain mean generation times that were accurate within two or three hours, especially when only five lines were considered. For example, if an organism had divided at the end of six and one half hours after isolation, the division would not have been counted until the end of the second six-hour period, and its generation time would go on record as twelve hours—an error of almost six hours or almost 100 per cent. of its true generation time of six and one half hours. In view of the small number of organisms involved, the time between observations in such isolation experiments should not be more than a fraction of the generation time of the organisms under observation, and should be much less than the expected difference which is to be measured. Since, in this case, the periods between observations were almost equal to the generation time, the value of the mean is accurate only within two or three hours, which is 33–50 per cent. of its probable true value. Therefore it is very unlikely that the 40 per cent. difference (2.4 hours) which Petersen calculated between one- and four-animal cultures has any significance at all except perhaps to show that the data which she chose from Myers' paper were entirely insufficient for such an analysis. It is also very difficult to understand how she could consider these two 16-drop series more important than the other ten sets which were performed with the same material under similar conditions, and yet gave opposite results. It is evident, therefore, that the only results of Myers (1927) which seem to indicate an allelocatalytic effect are not significant and that the interpretation placed upon these experiments by Peterson (1929) can not be valid. In view

of his results, Myers' own conclusions are logical, are supported by his evidence, and are opposed to the autocatalytic theory.

Robertson (1921*b*), in a series of experiments in which he isolated single ciliates and pairs of ciliates, chosen at random from young parent cultures, into the same volume of medium, found that, instead of twice as many organisms in the two-cell as in the single-cell cultures, there were from three to six times as many at the end of his observations. In isolations made from parent cultures over three days old, there were 2 to 2.7 as many in the two-cell cultures. Since all initial cells were isolated at random from mass cultures, the time since the last division was unknown, and the differences in the ages of the isolated cells might have been almost as much as the generation time of the organism. It can be shown also that the differences obtained by Robertson are not significant and are easily interpreted on a basis other than allelocatalysis.

Cutler and Crump (1924) found that cultures of *Colpidium* with few bacteria showed low division rates and that the organisms showed definite signs of hunger, while cultures with numerous bacteria showed a very high division rate. Other workers have tried unsuccessfully to obtain bacteria-free ciliates which would continue to reproduce. Peters (1921) believed that he was successful, but Cutler and Crump (1924) showed that his cultures were probably contaminated with a small bacillus. Parpart (1928) was successful in freeing *Paramecium* of bacteria, as were also Hargitt and Fray (1917) and Phillips (1922). Parpart makes no mention whatsoever of division in such "sterile" cultures, although he states that the organisms lived as long as five days. The only possible conclusion is that *Paramecium* does not reproduce in sterile culture medium. Hargitt and Fray (1917) and Phillips (1922) freed specimens of *Paramecium* from bacteria and then fed them known pure cultures of bacteria. Since the free-living ciliates have not been known to live without bacteria, and since there is a definite relationship between the number of bacteria and the division rate of ciliates, it would seem probable that the slow multiplication in Robertson's (1921*b*) one-cell cultures was due to lack of food in the form of bacteria.

The difference in growth rate between one- and two-animal

cultures shown in Robertson's experiments is not significant because variations obtained in his one-cell cultures are greater than the differences which are accorded to allelocatalysis. Some of his experiments, performed under supposedly identical conditions, are reanalyzed below in Table I.; since he quotes them in several publications (1921*a*, 1923, 1924*a*) they may be accepted as typical experiments. The data for the second and third columns of the table were taken from Robertson's graph.

TABLE I.

Culture Number.	Number present at First Observation.	Hours to First Observation.	Generations.	Average Generation Time to First Observation (in Hours).
238*A*	13	22	3½ approx.	6.3
240*A*	8	23	3	7.7
237*A*	8	22	3	7.3
242*A*	4	24	2	12.0

These generation times of various one-animal cultures of the *A* series show a variation of 5.7 hours, *e.g.*, culture 238*A* had an average generation time of 6.3 hours, and culture 242*A* a generation time of 12 hours. This is a variation of ± 31 per cent. from the median of 9.1 hours in cultures that, so far as known, were maintained under identical conditions.

Another of Robertson's experiments (Table II.) which he often quotes (Robertson, 1923, 1924*a*, *c*) as a proof of the allelocatalytic effect, may be compared with the data of Table I.

TABLE II.

Culture.	Initial Number.	24 hrs.	48 hrs.	Generations.	Average Gen. Time.
310*A*	1	2	16	4	12 hours
311*A*	2	3	120	6	8 hours

If the numbers of ciliates in cultures 310*A* and 311*A* are compared at the end of 48 hours, it is seen that there are 7.5 times as many cells in the two-animal culture. The average generation times are twelve hours for the one-animal culture and slightly more than eight hours for the two-animal culture. This is a dif-

ference of almost four hours, or a variation of ± 20 per cent. from the median of ten hours. Since Robertson's series of one animal cultures (Table I.), subjected to identical conditions, shows a variation of ± 31 per cent., it is impossible to consider this variation of ± 20 per cent. as being the result of any difference in division rate due to allelocatalysis. It is well within the normal variation and need not be accounted for otherwise.

Yocom (1928) isolated single individuals of *Oxytricha* into different amounts of sterile medium. He found a 14 per cent. higher division rate in the cultures of smaller volume and interpreted this as evidence of a catalyst of growth. In view of the previous discussion of bacterial food supply in this paper it is easy to see how such a small difference could arise. The food supply was less concentrated in the cultures of larger volume, and thus less readily available; hence the ciliates did not divide as rapidly as in the cultures of smaller volume.

Petersen (1929), in numerous isolation experiments with *Paramecium*, obtained results which are contradictory in that some experiments favor Robertson's view, while others are directly opposed to the theory of autocatalysis. In small volumes no acceleration was noticed in two-animal cultures, and washing produced no noticeable difference in division rate. However, when animals were isolated, washed or unwashed, singly, in two's, and in four's, into twenty drops of bacterized medium, the highest rate was observed in the four-animal cultures and the lowest in the one-animal cultures. Similar results were obtained with forty-drop cultures. Since Petersen's results argue both for and against allelocatalysis, an explanation of such contradiction must be found before her observations may be accepted as either supporting or opposing Robertson's theory.

The results of the writer's investigations offer no evidence for allelocatalysis. Except in one doubtful case, the cultures of higher concentration showed a growth rate equal to or less than that of the more dilute cultures. If an accelerating factor such as a "catalyst of growth" had been exuded during divisions, the higher concentration of this catalyst in the more concentrated cultures would have accelerated their growth, *especially after the first few divisions*. Since the growth rate was not accelerated

during the first few days it must be concluded that no such "catalyst of growth" was present.

In most cases in which growth rate of the more concentrated cultures was less than that of the more dilute the difference became more and more pronounced as the culture grew older. This indicates an inhibiting factor in the more concentrated cultures, especially after the first few days. Part of the later differences are, of course, exaggerations due to the geometrical method of increase of early differences, but the causative agent of any difference, early or late, is unknown. The fact that most of the more concentrated cultures did not grow as rapidly as the more dilute is perhaps to be explained as due to a more rapidly diminishing food supply or a more rapid accumulation of waste products in the concentrated cultures or, more likely, to both these factors. This explanation is in accord with the results of Woodruff (1913), Cutler and Crump (1923*a*, *b*), Greenleaf (1926) and others on ciliates.

At the present time Robertson's theory of allelocatalysis appears to be upheld only by some of the experiments of Petersen (1929). The theory is opposed by the investigations of Woodruff (1911, 1913), Cutler and Crump (1923*a*, *b*, 1924, 1925), Greenleaf (1924, 1926), Calkins (1926), Myers (1927), some of the results of Petersen (1929), and by the investigations of the writer. In view of this bulk of evidence against the theory, and in view of the fact that the most striking results of Robertson are shown to be insignificant, the only conclusion to be drawn is that an allelocatalytic effect is not present in protozoan cultures.

Summary.

1. A method of obtaining accurate growth curves of euglenoid mass cultures is described and its efficiency demonstrated.

2. It is shown that under the conditions described no allelocatalytic effect is present in cultures of *Euglena* sp., and it is also shown that data of other workers which have been used to support the allelocatalytic theory do not support it but indicate the absence of any allelocatalytic effect.

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